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EP-A- 0 300 988

PROC. NATL ACAD. SCI. USA, vol. 84, April
1987, pages 1886-1890; G.J. BROZE Jr. et al:
"Isolation of the tissue factor inhibitor pro-
duced by HepG2 hepatoma cells"

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THE JOURNAL OF BIOLOGICAL CHEMISTRY,
vol. 263, no. 13, 5th May 1988, pag. 5
6001-6004, The American Society of Bio-
chemistry and Molecular Biology, Inc., US;
T.-C. WUN et al: "Cloning and characterization
of a cDNA coding for the lipoprotein-
associated coagulation inhibitor shows that
it consists of three tandem Kunitz-type in-
hibitory domains"

BIOLOGICAL AB-
STRACTS/REPORTS-REVIEWS-MEETINGS,
vol. 35, 1988, abstract no. 35060091; T.J. GI-
RARD et al: "Cloning and sequencing of a
complementary DNA for the human
lipoprotein associated coagulation inhibitor"

Description

Background of the Invention

5 This invention relates to a coagulation inhibitor known as tissue factor inhibitor (TFI) and alternatively as lipoprotein associated coagulation inhibitor (LACI). More particularly, the invention relates to a cDNA clone representing essentially the full size TFI.

The coagulation cascade that occurs in mammalian blood comprises two distinct systems - the so-called intrinsic and extrinsic systems. The latter system is activated by exposure of blood to tissue thromboplastin (Factor III), hereinafter referred to as tissue factor (TF). Tissue factor is a lipoprotein that arises in the plasma membrane of many cell types and in which the brain and lung are particularly rich. Upon coming into contact with TF, plasma Factor VII or its activated form, Factor VII_a, forms a calcium-dependent complex with TF and then proteolytically activates Factor X to Factor X_a, and Factor IX to Factor IX_a.

15 Early studies concerning the regulation of TF-initiated coagulation showed that incubation of TF (in
crude tissue thromboplastin preparations) with serum inhibited its activity in vitro and prevented its lethal
effect when it was infused into mice. Extensive studies by Hjort, Scand. J. Clin. Lab. Invest. 9, Suppl. 27,
76-97 (1957), confirmed and extended previous work in the area, and led to the conclusion that an inhibitory
moiety in serum recognized the Factor VII-TF complex. Consistent with this hypothesis are the facts that
20 the inhibition of TF that occurs in plasma requires the presence of Ca^{2+} (which is also necessary for the
binding of Factor VII/VII_a to TF) and that inhibition can be prevented and/or reversed by chelation of divalent
cations with EDTA. More recent investigations have shown that not only Factor VII_a but also catalytically
active Factor X_a and an additional factor are required for the generation of TF inhibition in plasma or serum.
25 See Broze and Miletich, Blood 69, 150-155 (1987), and Sanders et al., Ibid. 66, 204-212 (1985). This
additional factor, defined herein as tissue factor inhibitor (TFI), and alternatively as lipoprotein associated
coagulation inhibitor (LACI), is present in barium-absorbed plasma and appears to be associated with
lipoproteins, since TFI functional activity segregates with the lipoprotein fraction that floats when serum is
30 centrifuged at a density of 1.21 g/cm³. According to Broze and Miletich, supra, and Proc. Natl. Acad. Sci.
USA 84, 1886-1890 (1987), HepG2 cells (a human hepatoma cell line) secrete an inhibitory moiety with the
same characteristics as the TFI present in plasma.

In copending application US Ser. No. 77,366, filed July 23, 1987, (priority document of EP 0 300 988), a purified tissue factor inhibitor (TFI) is disclosed which was secreted from HepG2 cells. It was found to exist in two forms, a TFI_1 , migrating at about 37-40,000 daltons and a TFI_2 at about 25-26,000 daltons, as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). A partial N-terminal amino acid sequence for the TFI was assigned as:

35 amino acid sequence for the TFI was assigned as:

1 X-X-Glu-Glu-Asp-Glu-Glu-His-Thr-Ile-Ile-Thr-Asp-Thr-Glu-
 16 Leu-Pro-Pro-Leu-Lys-Leu-Met-His-Ser-Phe-(Phe)-Ala 27

45 wherein X-X had not been determined. The disclosure of said application is incorporated herein by reference.

Brief Description of the Invention

50 In accordance with the present invention, the complete coding sequence of a cDNA clone representing essentially the full size tissue factor inhibitor (TFI) has been developed.

Initially, human placental and fetal liver λ gt11 cDNA libraries were screened with a rabbit polyclonal antiserum raised against a purified TFI. Immunologically positive clones were further screened for 125 I-Factor X₀ binding activity. Seven clones were obtained which were immunologically and functionally active.

55 The longest clone, placental-derived λ P9, was 1.4 kilobases (kb) long while the other six were 1.0 kb in length. Partial DNA sequencing showed the 1.0 kb clones to have sequences identical to part of the longer 1.4 kb clone. Nucleotide sequence analysis showed that λ P9 consisted of a 1432 basepair (bp) cDNA insert that includes a 5'-noncoding region of 133 bp, an open reading frame of 912 bp, a stop codon, and a 3'-

noncoding region of 384 bp.

The cDNA sequence encodes a 31,950 Dalton protein of 276 amino acids which includes 18 cysteines and 7 methionines. The translated amino acid sequence shows that a signal peptide of about 28 amino acids precedes the mature TFI protein. It will be understood that the "mature" TFI is defined to include both

5 TFI and m thionyl TFI by virtue of the ATG translational codon in the λ P9 clone described herein.

There are three potential N-linked glycosylation sites in the TFI protein with the sequence Asn-X-Ser/Thr, wherein X can be any of the common 20 amino acids. These sites are at amino acid positions Asn 145, Asn 195, and Asn 256, when the first methionine after the 5'-noncoding region is assigned amino acid position + 1.

10 The translated amino acid sequence of TFI shows several discernible domains, including a highly negatively charged N-terminal, a highly positively charged carboxy-terminal, and an intervening portion consisting of 3 homologous domains with sequences typical of Kunitz-type enzyme inhibitors. Based on a homology study, TFI appears to be a member of the basic protease inhibitor gene superfamily.

15 The original source of the protein material for developing the cDNA clone λ P9 was human placental tissue. Such tissue is widely available after delivery by conventional surgical procedures. The λ gt11 (lac5 nin5 c1857 S100) used herein is a well-known and commonly available lambda phage expression vector. Its construction and restriction endonuclease map is described by Young and Davis, Proc. Natl. Acad. Sci. USA 80, 1194-1198 (1983).

20 Northern blot analysis showed that the following liver-derived cell lines: Chang liver, HepG2 hepatoma, and SK-HEP-1 hepatoma, all contained 2 major species of mRNA (1.4 and 4.4 kb) which hybridized with the TFI cDNA.

25 The cloning of the cDNA for TFI and development of its entire protein sequence and structural domains as disclosed herein permits detailed structure-functional analyses and provides a foundation for study of its biosynthetic regulations. The invention thus is important to medical science in the study of the coagulation cascade with respect to agents which are able to inhibit Factor X_a and Factor VII_a /TF enzymatic complex.

Detailed Description of the Invention

30 While the specification concludes with claims particularly pointing out and distinctly claiming the subject matter regarded as forming the present invention, it is believed that the invention will be better understood from the following detailed description of preferred embodiments of the invention taken in conjunction with the appended drawings, in which:

35 FIG. 1 shows the screening of λ gt11 clones with 125 I-Factor X_a . Cloned phage lysates (0.1 ml) were spotted on a nitrocellulose paper by suction using a dot blot apparatus. The nitrocellulose paper was then probed with 125 I-Factor X_a and autoradiographed as described hereinafter. The clones that appear as dark spots are positive clones that bind 125 I-Factor X_a . Control λ gt11 (lower right corner) and other clones do not bind 125 I-Factor X_a .

40 FIG. 2 shows a partial restriction map and sequencing strategy for the λ P9 inserts. The scale at the bottom indicates the nucleotide position. The thick bar represents the coding region. The thin bars represent 5'- and 3'-noncoding regions. The restriction endonuclease sites were confirmed by digestion. The arrows show the overlapping M13 clones used to sequence the cDNA.

45 FIG. 3 shows the nucleotide sequence and translated amino acid sequence of the human TFI cDNA. Nucleotides are numbered on the left and amino acids on the right. The underlined sequences have been independently confirmed by amino acid sequence analysis of the purified TFI protein and two V_8 protease + trypsin digested peptides. Amino acid + 1 was assigned to the first methionine after a stop codon of the 5'-noncoding region. Potential N-linked glycosylation sites are marked by asterisks.

50 FIG. 4 is a graphical representation which shows the charge distribution of the amino acid sequence in TFI. Charges are calculated from the first residue to the i-th residues and displayed at the i-th residue. Thus the value of the i-th position is the summation of all charges from the first residue to the i-th residue and the difference of the charges between the i-th and j-th residue ($j > i$) is the net charge of the fragment from i-th to j-th residue.

55 FIG. 5 is a graphical representation which shows the hydrophobicity profile of TFI. The hydrophobicity profile was analyzed by a computer program whereby the hydrophobicity index of the amino acid residues is defined as the depth to which an amino acid residue is buried inside a protein (from X-ray crystallographic data) [Kidera et al., J. Protein Chem. 4, 23-55 (1985)]. The hydrophobicity profile along the sequence was smoothed using the program ICSSCU in IMSL Library [IMSL Library Reference Manual, 9th ed., Institute for Mathematical and Statistical Subroutine Library, Houston, Texas (1982)].

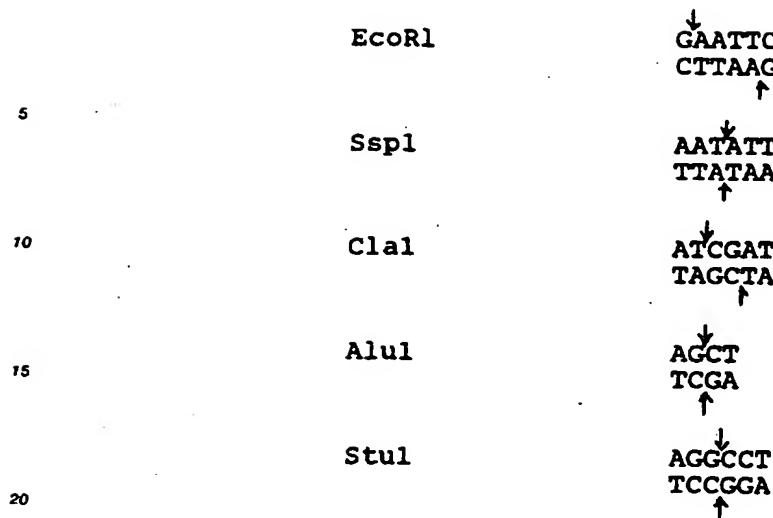
FIG. 6 shows an alignment of the basic protease inhibitor domains of TFI with other basic protease inhibitors. All the sequences except TFI were obtained from the National Biomedical Research Foundation Protein Sequence Database (Georgetown University, Washington, D.C., release 13, Jun 1987). 1. Bovine basic protease inhibitor precursor; 2. Bovine colostrum trypsin inhibitor; 3. Bovine serum basic protease inhibitor; 4. Edible snail iso-inhibitor K; 5. Red sea turtle basic protease inhibitor (only amino acids 1-79 presented); 6. Western sand viper venom basic protease inhibitor I; 7. Ringhals venom basic protease inhibitor II; 8. Cape cobra venom basic protease inhibitor II; 9. Russell's viper venom basic protease inhibitor II; 10. Sand viper venom basic protease inhibitor III; 11. Eastern green mamba venom basic protease inhibitor I homolog; 12. Black mamba venom basic protease inhibitor B; 13. Black mamba venom basic protease inhibitor E; 14. Black mamba venom basic protease inhibitor I; 15. Black mamba a venom basic protease inhibitor K; 16. β -1-Bungarotoxin B chain (minor); 17. β -1-Bungarotoxin B chain (major); 18. β -2-Bungarotoxin B chain; 19. Horse inter- α -trypsin inhibitor [amino acids 1-57(1); 58-123(2)]; 20. Pig inter- α -trypsin inhibitor [amino acids 1-57(1); 58-123(2)]; 21. Bovine inter- α -trypsin inhibitor [amino acids 1-57(1); 58-123(2)]; 22. Human α -1-microglobulin/inter- α -trypsin inhibitor precursor [amino acids 227-283(1); 284-352(2)]; 23. TFI [amino acids 47-117(1); 118-188(2); 210-280(3)]. Gaps were included in 16, 17, 18 to achieve best alignment. Standard one letter codes for amino acids are used.

FIG. 7 shows the Northern blot analysis of RNAs from 3 liver-derived cell lines. Ten μ g of poly(A)⁺ RNA were used per lane. Lane 1, Chang liver cell; lane 2, SK-HEP-1 hepatoma cell; lane 3, HepG2 hepatoma cell.

Standard biochemical nomenclature is used herein in which the nucleotide bases are designated as adenine (A); thymine (T); guanine (G); and cytosine (C). Corresponding nucleotides are, for example, deoxyguanosine-5'-triphosphate (dGTP). As is conventional for convenience in the structural representation of a DNA nucleotide sequence, only one strand is shown in which A on one strand connotes T on its complement and G connotes C. Amino acids are shown either by three letter or one letter abbreviations as follows:

Abbreviated Designation		Amino Acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Commonly available restriction endonucleases described herein have the following restriction sequences and (indicated by arrows) cleavage patterns:



In order to illustrate specific preferred embodiments of the invention in greater detail, the following exemplary laboratory preparative work was carried out.

26 Example 1

Materials

30 Human placental and fetal liver cDNA libraries were obtained from Clonetech. The protoblot immunoscreening kit was purchased from Promega Biotech. Restriction enzymes were from New England Biolabs. Calf intestine alkaline phosphatase, T4 DNA ligase, DNA polymerase I (Klenow), exonuclease III and S1 nuclease were from Boehringer Mannheim. dNTPs were from P. L. Biochemicals. 5'-{ α -³⁵S}-thio-dATP (600 Ci/mmol) was from Amersham. Sequencing kit (Sequenase) was from United States Biochemicals. Chang liver cells (ATCC CCL 13) and HepG2 hepatoma cells (ATCC HB 8065) were obtained from the American Type Culture Collection. SK-HEP-1 hepatoma cells were originally derived from a liver adenocarcinoma by G. Trempe of Sloan-Kettering Institute for Cancer Research in 1971 and are now widely and readily available.

35 ¹²⁵I-Factor X₀ was prepared by radiolabeling using Iodo-gen. The specific activity was 2000 dpm/ng. Greater than 97% of radioactivity was precipitable with 10% trichloroacetic acid (TCA). The iodinated protein retained >80% of their catalytic activity toward Spectrozyme X₀ (American Diagnostica product).

40 An anti-TFI-Ig Sepharose® 4B column was prepared as follows: A peptide (called TFI-peptide) containing a sequence corresponding to the amino acid sequence 3-25 of the mature TFI was synthesized using Biosystem's solid phase peptide synthesis system. The TFI-peptide (5 mg) was conjugated to 10 mg of Keyhole limpet hemocyanin by glutaraldehyde. Two New Zealand white rabbits were each immunized by intradermal injection with a homogenate containing 1 ml of Freund complete adjuvant and 1 ml of conjugate (200 μ g of TFI-peptide). One month later the rabbits were each boosted with a homogenate containing 1 ml of Freund incomplete adjuvant and 1 ml of conjugate (100 μ g of conjugate). Antiserum was collected each week for 3 months and booster injections were performed monthly. To isolate specific antibody against TFI-peptide, the antiserum was chromatographed on a TFI-peptide Sepharose 4B column. The column was washed with 10 volumes of PBS (0.4 M NaCl-0.1 M benzamidine-1% Triton® X-100) and the same solution without Triton X-100. The antibody was eluted with 0.1 M glycine/HCl, pH 2.2, immediately neutralized by adding 1/10 volume of 1 M Tris-OH and dialyzed against saline solution. The isolated antibody was coupled to cyanogen bromide activated Sepharose 4B by the manufacturer's (Pharmacia) method and used to isolate TFI from the c II culture medium.

45 Chang liver cell was cultured by the method described previously by Broze and Millich, Proc. Natl. Acad. Sci. USA 84, 1886-1890 (1987). The conditioned medium was chromatographed on the anti-TFI-Ig Sepharose 4B column. The column was washed with 10 volumes of PBS-1% Triton X-100 and PBS. Th-

bound TFI was eluted with 0.1 M glycine/HCl, pH 2.2. The immunoaffinity isolated TFI was further purified by preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (Savant apparatus). Amino acid analysis of the final product showed the same amino terminal sequence as the TFI isolated from H pG2 cells as described in copending application, US Ser. No. 77,366, filed July 23, 1987, (priority document of

5 EP 0 300 988). The isolated Chang liver TFI was then used to immunize rabbits by the immunization protocol described above. The antiserum obtained had a titer of about 100 µg/ml in the double immunodiffusion test. This antiserum was used in the immuno-screening of λgt11 cDNA libraries.

Methods

10

Isolation of cDNA clones. Methods for screening the placental and fetal liver cDNA libraries with antibody, plaque purification, and preparation of λ-phage lysate and DNA were as described by Wun and Kretzmer, *FEBS Lett.* 1, 11-16 (1987). The antiserum was pre-adsorbed with BNN97 λgt11 lysate and diluted 1/500 for screening the library.

15

Screening of factor X_a binding activity

Recombinant proteins induced by isopropyl-β-thiogalactoside from immuno-positive λ-phage isolates or from control λgt11 were screened for Factor X_a binding activity. The λ-phage lysates (0.1 ml) were filtered 20 through a nitrocellulose paper using a dot-blot apparatus (Bio Rad). The nitrocellulose paper was then immersed and agitated in a phosphate buffered saline containing 5 mg/ml bovine serum albumin and 2.5 mg/ml bovine gamma globulin at room temperature for 1 h. The solution was replaced with ¹²⁵I-Factor X_a - (1.0 x 10⁶ cpm/ml) dissolved in the same solution supplemented with 0.1 mg/ml heparin and the agitation continued for another hour. The nitrocellulose paper was then washed with phosphate buffered saline 25 containing 0.05% Tween® 20. The washing buffer was changed every 5 min., 4 times. The nitrocellulose paper was then air-dried and prepared for autoradiography using Kodak XAR film. The film was developed after 1 week exposure.

Preparation of poly(A)⁺ RNA and Northern blotting. Total RNAs were prepared from cultured Chang liver cell, HepG2 hepatoma cell and SK-HEP-1 hepatoma cell using the sodium perchlorate extraction method of Lizardi, and Engelberg, *Anal. Biochem.* 98, 116-122, (1979). Poly(A)⁺ RNAs were isolated by batch-wise 30 adsorption on oligo(dT)-cellulose (P-L Biochemical, type 77F) using the procedure recommended by the manufacturer. For Northern blot analysis, 10 µg each of poly(A)⁺ RNA was treated with glyoxal [Thomas, *Methods Enzymol.* 100, 255-268 (1983)] and subjected to agarose gel electrophoresis in a buffer containing 10 ml sodium phosphate, pH 7.0. Bethesda Research Laboratory's RNA ladder was used as a molecular 35 weight marker. The RNAs were transblotted onto a nitrocellulose paper which was then baked at 80° for 2 h. The insert DNA of λP₃ clone was radiolabeled with ³²P by nick translation and used as a probe [Maniatis et al.,

Molecular Cloning: A Laboratory Model, Cold Spring Laboratory, Cold Spring Harbor, N.Y., (1982)]. The blot was hybridized with 5 x 10⁶ cpm of the probe in 5 ml of a solution containing 50% formamide, 5X SSC, 50 40 mM sodium phosphate, pH 7.0, 250 µg/ml denatured salmon sperm DNA, and 1X Denhardt's solution at 42° for 16 h. The filter was washed in 0.1% sodium dodecylsulfate (SDS), 2X SSC at room temperature 3 times, each time 5 min., and in 0.1% SDS, 0.2 X SSC at 50° twice, each 5 min. The nitrocellulose paper was then air dried, autoradiographed for 3 days at -70° using Kodak XAR-5 film and intensifying screen.

Other recombinant DNA methods. Preparation of cloned Agt11 DNA, subcloning in pUC19 plasmid and 45 M13mp18 vector, generation of deletion by exonuclease III digestion and DNA sequencing by dideoxy method [Sanger et al., *Proc. Natl. Acad. Sci. USA* 83, 6776-6780 (1977)], were performed as described by Wun and Kretzmer, *supra*.

The program FASTP written by Lipman and Pearson, *Science* 227, 1435-1441 (1985), was used to identify homologous families of proteins from National Biomedical Research Foundation Sequence Data 50 Bank (release 13, June 1987) and to align the sequences within the homologous family.

RESULTS

Screening of cDNA libraries

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A number of cell lines were screened for the presence of TFI in the conditioned media and it was found that several liver-derived cell lines, Chang liver, HepG2 hepatoma, and SK-HEP-1 hepatoma secreted TFI in culture. Initially, an antiserum against TFI was used to screen a human fetal liver λgt11 cDNA library (10⁶

plaque forming units), and 15 immunologically positive clones were obtained. Subsequently, the same method was used to screen a placental λgt11 cDNA library. Out of 10^6 plaque forming units, 10 immunologically positive clones were obtained. These clones were plaque purified and the lysates of the purified clones were tested for the functional activity of TFI. The isopropylthiogalactosid induced phag 5 lysates were absorbed on the nitrocellulose paper and screened for the ^{125}I -Factor X_a binding activity. Figure 1 demonstrates that some of these immunologically positive clones showed the ability to bind the ^{125}I -Factor X_a on the nitrocellulose paper. In all, 3 out of 15 immunologically positive fetal liver clones, and 4 out of 10 immunologically positive placental clones showed ^{125}I -Factor X_a binding activity. These immunologically and functionally positive clones were digested with EcoR1 and the size of the inserts were 10 estimated by gel electrophoresis. One clone from placental library (λP9) had an insert of approximately 1.4 kb, while all the other clones contain inserts of approximately 1.0 kb. Partial DNA sequencing has showed that 1.0 kb clones contain sequences identical to part of the longer 1.4 kb placental clone (λP9). The λP9 was therefore selected for complete sequencing.

15 Nucleotide sequence and predicted protein sequence of TFI cDNA isolate

The λP9 clone was subjected to restriction mapping, M13 subcloning and sequencing by the strategy shown in Figure 2. The entire sequence was determined on both strands by the exonuclease III deletion method [Henikoff, *Gene* 28, 351-359 (1984)] and found to consist of 1432 bases in length. The sequence is 20 shown in Figure 3. It contains a 5'-noncoding region of 133 bases, an open reading frame of 912 nucleotides, and a 3'-noncoding region of 387 nucleotides. The first ATG occurs at nucleotide 134 in the sequence TAGATGA which was closely followed by a second ATG at nucleotide 146 in the sequence ACAATGA. These are possibly the initiation sequences, although they differ from the proposed consensus sequence for initiation by eukaryotic ribosome, ACCATGG [Kozak, *Cell* 44, 283-292 (1986)]. Twenty-eight 25 amino acids precede a sequence corresponding to the N-terminal of the mature protein. The length and composition of the hydrophobic segment of these 28 amino acids are typical of signal regions [Von Heijne, *Eur. J. Biochem.* 133, 17-21 (1983); *J. Mol. Biol.* 184, 99-105 (1985)]. A signal peptidase possibly cleaves at Ala₂₈-Asp₂₉ to give rise to a mature protein. The sequence predicted for mature TFI consists of 276 amino acids that contains 18 30 cysteine residues and 7 methionines. The calculated mass of 31,950 Daltons based on the deduced protein sequence for mature TFI is somewhat lower than the 37-40 kDa estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis of isolated protein, and the difference probably reflects the contribution of glycosylation to the mobility of the natural protein. The deduced protein sequence corresponding to the 35 mature protein contains 3 potential N-linked glycosylation sites with the sequence Asn-X-Thr/Ser (amino acid positions 145, 195, and 256). Amino acid sequence analysis of purified whole TFI and two isolated proteolytic fragments match exactly the protein sequence deduced from cDNA sequence (Figure 3, underlined), indicating the isolated cDNA clone encodes TFI. The 3'-noncoding region is A+T rich (70% A+T). Neither consensus polyadenylation signal, AATAAA [Proudfoot and Brownlee, *Nature* 252, 359-362 40 (1981)] nor the poly A tail was found in this clone, possibly due to artefactual loss of part of 3' terminal portion during construction of the library.

Charge distribution, hydrophobicity/hydrophilicity, and internal homology

The translated amino acid sequence of the TFI contains 27 lysines, 17 arginines, 11 aspartic acids, and 45 25 glutamic acids. The charge distribution along the protein is highly uneven as shown in Figure 4. The signal peptide region contains 2 positively charged lysine with 26 neutral residues. The amino-terminal region of the mature protein contains a highly negatively charged stretch. Six of the first 7 residues are either aspartic acid or glutamic acid which are followed closely by two more negatively charged amino acids downstream before a positively charged lysine residue appears. The center portion of the molecule is 50 generally negatively charged. At the carboxy terminal, there is a highly positively charged segment. The amino acids 265 to 293 of TFI contain 14 positively charged amino acids including a 6-consecutive arginine + lysine residues.

The predicted hydrophilicity/hydrophobicity profile of TFI protein is shown in Figure 5. The signal peptide contains a highly hydrophobic region as expected. The rest of the molecule appears rather 55 hydrophilic.

The translated amino acid sequence of TFI contains several discernible domains. Besides the highly negatively charged N-terminal domain and the highly positively charged C-terminal domain, the center portion consists of 3 homologous domains which have the typical sequences of the Kunitz-type inhibitors

(see below).

Homology to other proteins

5 By searching the National Biomedical Research Foundation sequence data base, it was found that the N-terminal domain and C-terminal domain of TFI do not show significant homology to other known proteins. The 3 internal homologous domains, however, are each homologous to the sequences of other basic protease inhibitors including bovine pancreatic basic protease inhibitor (aprotinin), venom basic protease inhibitors, and inter- α -trypsin inhibitors (Figure 6). It is noteworthy that disulfide bonding structure is highly
10 conserved in all these inhibitors. Based on these homologies, it is clear that TFI belongs to the basic protease inhibitor gene superfamily.

Northern blotting

15 Poly(A) + RNAs were purified from TFI-producing liver-derived cell lines, Chang liver, HepG2 hepatoma, and SK-HEP-1 hepatoma cells. The poly (A) + RNAs were resolved by denaturing agarose gel electrophoresis, transferred onto a nitrocellulose paper and probed with 32 P-labeled TFI cDNA (λ P9). As shown in Figure 7, two major bands of hybridization were observed that corresponded to mRNAs of 1.4 kb and 4.4 kb in all three cell lines tested. Several other cell lines were tested which do not produce detectable
20 amounts of TFI and in which no hybridization with the probe was found. (data not shown).

Claims

25 1. cDNA sequence coding for mature human tissue factor inhibitor protein having the nucleotide sequence as shown in FIG. 3 of the drawings.

2. The cDNA sequence of Claim 1 characterized in that the cDNA is the human tissue factor inhibitor cDNA clone λ P9 characterized as shown in the restriction map in FIG. 2 of the drawings.

30 3. The cDNA sequence of any of Claims 1 or 2 encoding a protein having a mass of 37-40 kDaltons as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and having the amino acid sequence as shown in FIG. 3 of the drawings.

35 4. The cDNA sequence of any of Claims 1 or 2 encoding a protein which is the methionyl tissue factor inhibitor protein containing the amino acid sequence as shown in FIG. 3 of the drawings.

Patentansprüche

40 1. cDNA-Sequenz, die für ein reifes Humangewebsfaktorinhibitorprotein kodiert, mit der in Fig. 3 der Zeichnungen gezeigten Nucleotidsequenz.

2. cDNA-Sequenz nach Anspruch 1, dadurch gekennzeichnet, daß die cDNA der Humangewebsfaktorinhibitor-cDNA-Klon λ P9 ist, gekennzeichnet wie in der Restriktionskarte der Fig. 2 der Zeichnungen gezeigt.

45 3. cDNA-Sequenz nach einem der Ansprüche 1 oder 2, welche für ein Protein mit einer Masse von 37-40 kDalton kodiert, wie durch Natriumdodecylsulfat-Polyacrylamidelektrophorese bestimmt und mit der in Fig. 3 der Zeichnungen gezeigten Aminosäuresequenz.

50 4. cDNA-Sequenz nach einem der Ansprüche 1 oder 2, welche für ein Protein kodiert, das das Methionylgewebsfaktorinhibitorprotein ist, welches die in Fig. 3 der Zeichnungen gezeigte Aminosäuresequenz enthält.

Revendications

55 1. Séquence d'ADN codant une protéine mature qui est un inhibiteur du facteur tissulaire humain de la coagulation, et présentant la séquence de nucléotides indiquée sur la figure 3 des dessins annexés.

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2. Séquence d'ADNc conforme à la revendication 1, caractérisé en ce que cet ADNc est le clone λPG d'ADNc d'inhibiteur du facteur tissulaire humain de la coagulation, qui présent les caractéristiques indiquées dans la carte de restriction représentée sur la figure 2 des dessins annexés.
- 5 3. Séquence d'ADNc conforme à l'une des revendications 1 et 2, codant une protéine dont la masse, estimée d'après une électrophorèse en gel de polyacrylamide-dodécylsulfate de sodium, vaut de 37 à 40 kilodaltons et dont la séquence d'acides aminés est celle qui est représentée sur la figure 3 des dessins annexés.
- 10 4. Séquence d'ADNc conforme à l'une des revendications 1 et 2, codant une protéine qui est l'inhibiteur méthionylé du facteur tissulaire humain de la coagulation et qui contient la séquence d'acides aminés représentée sur la figure 3 des dessins annexés.

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FIG. 1

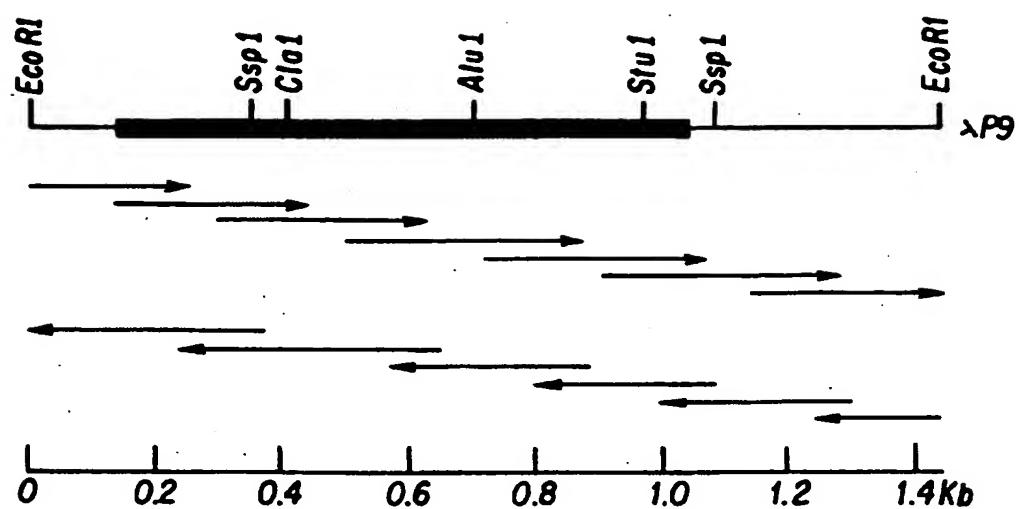


FIG. 2

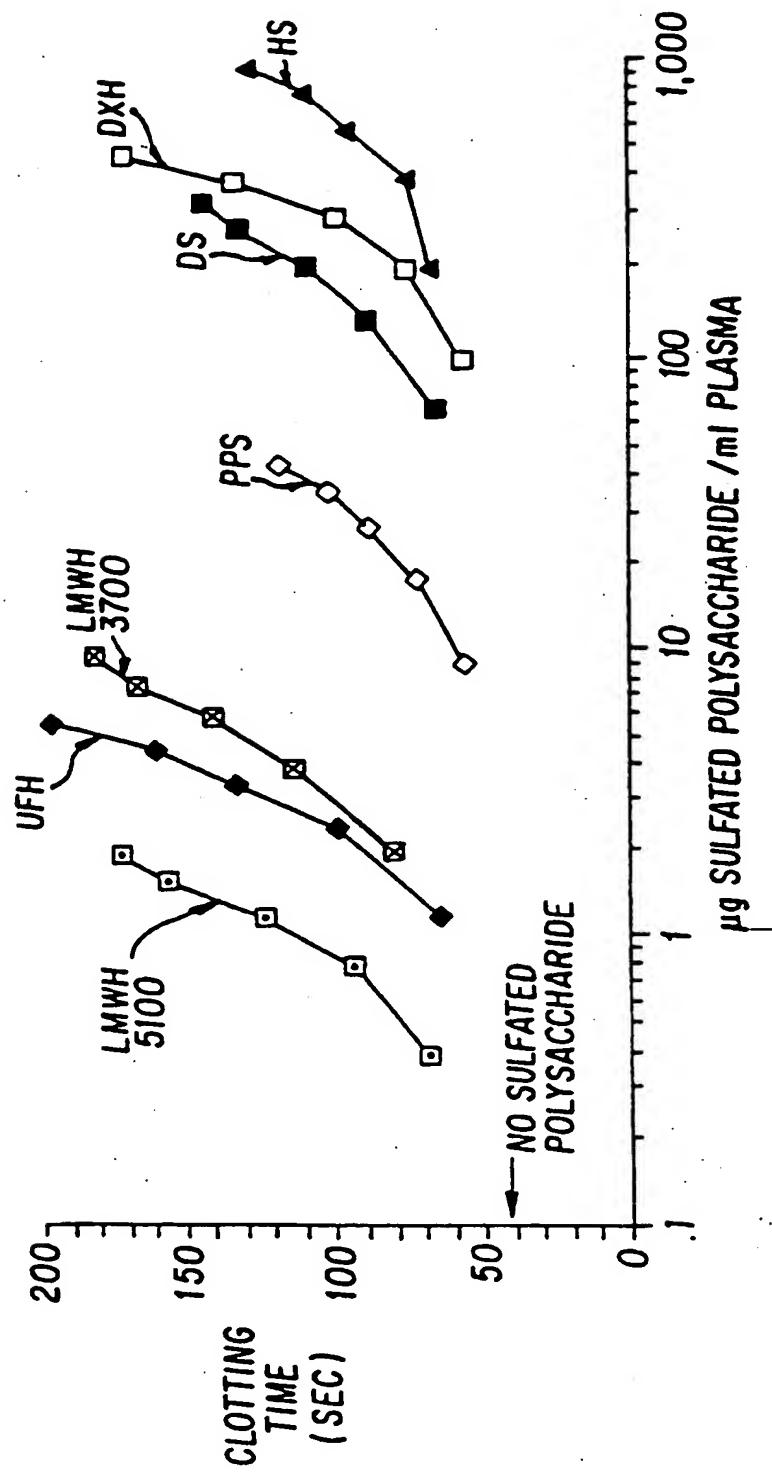


FIG. 6

FIG. 7A

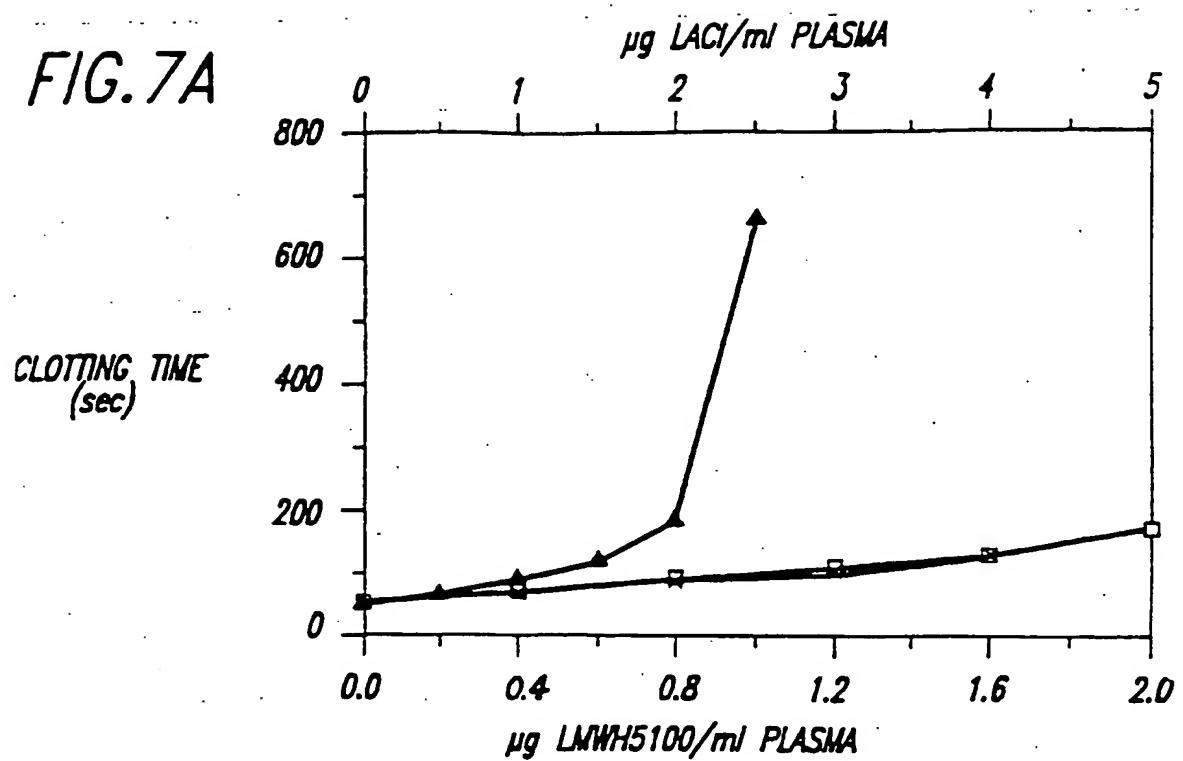


FIG. 7B

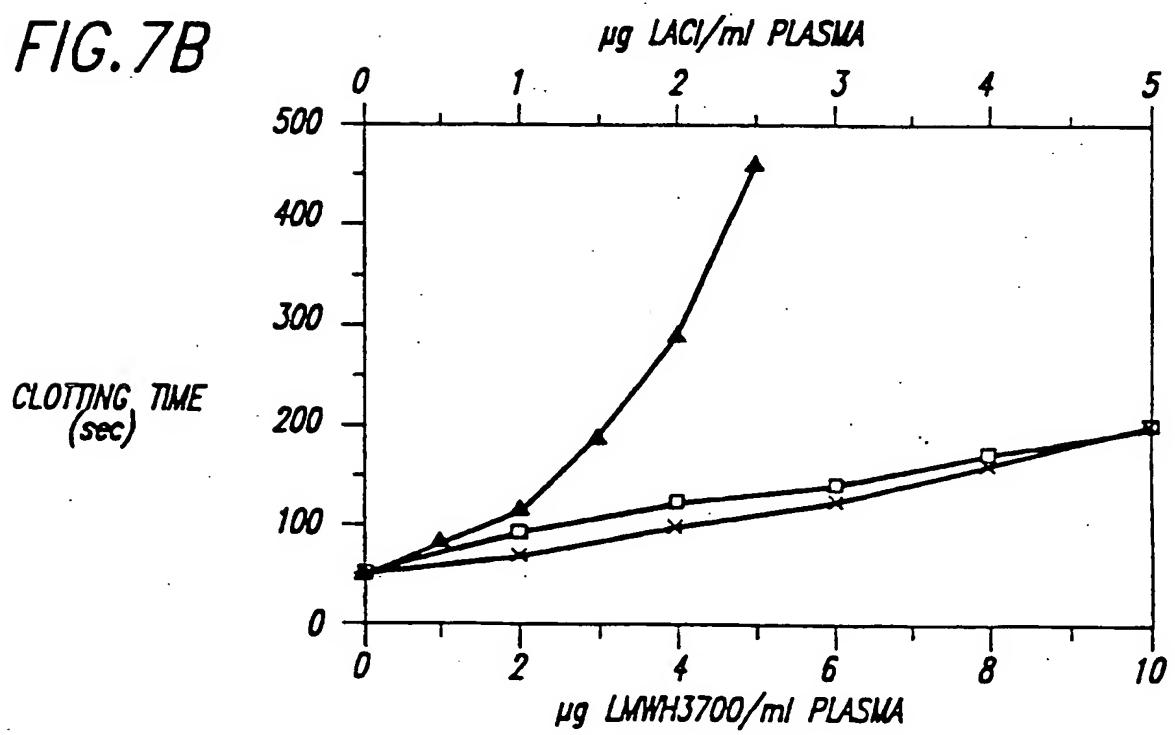


FIG. 7C

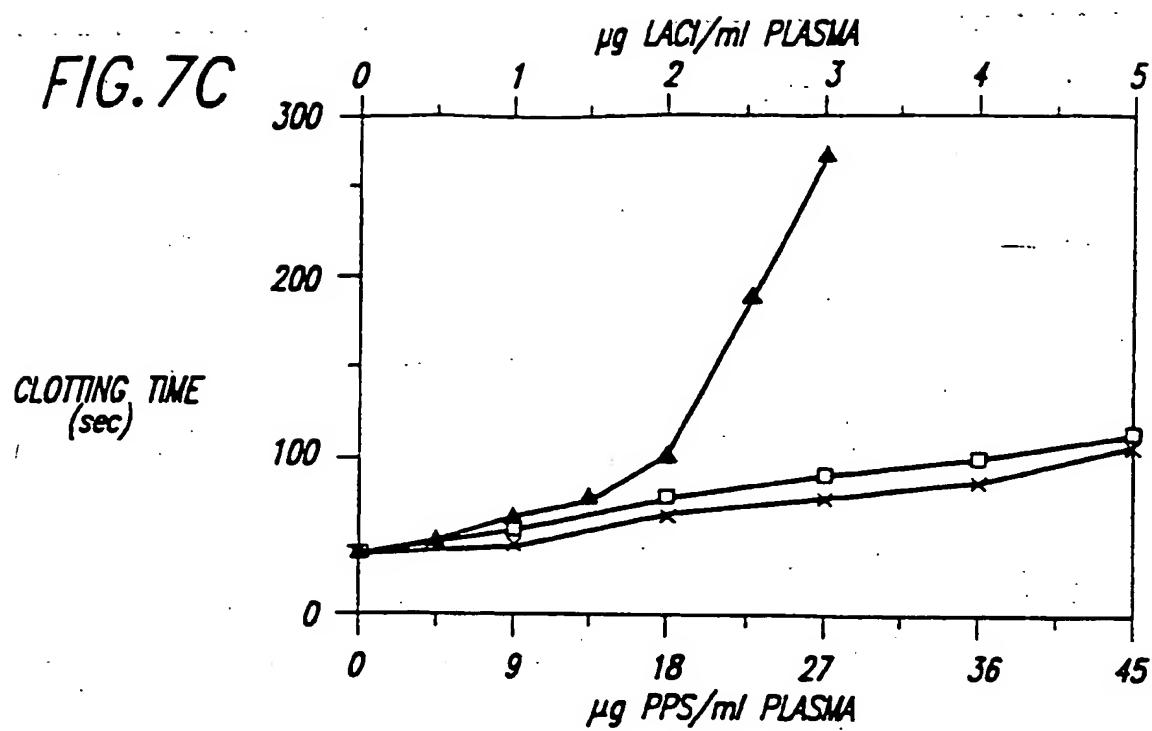


FIG. 7D

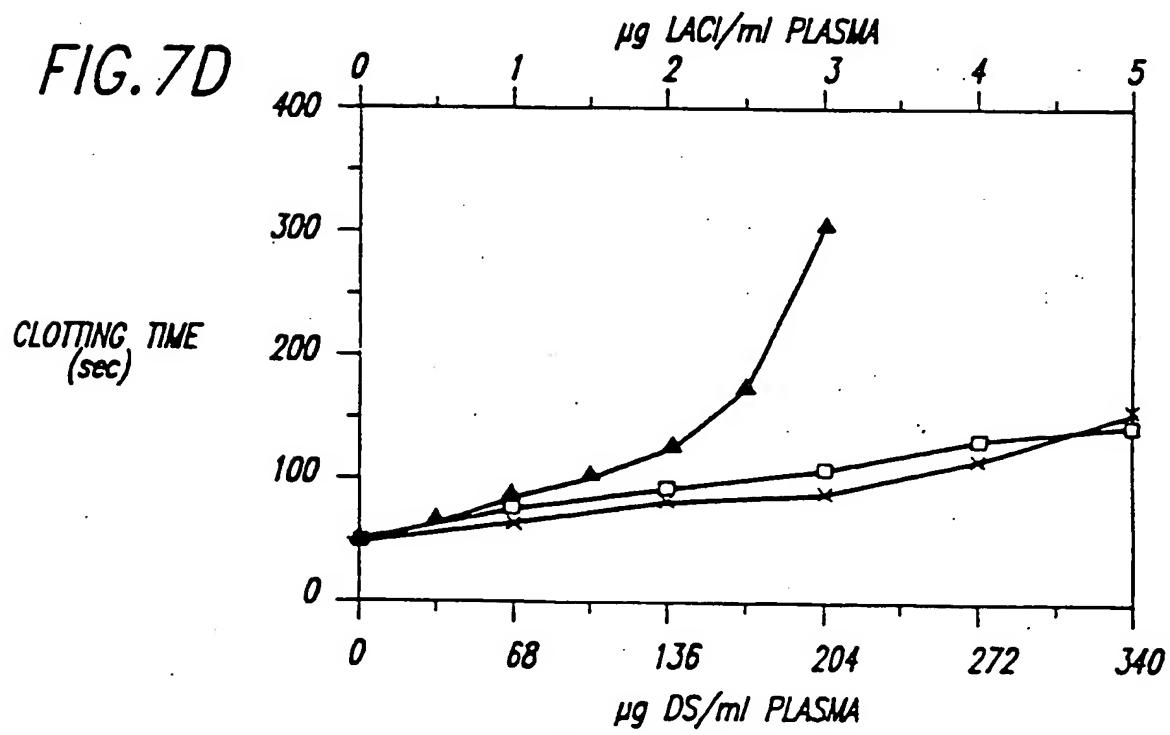


FIG. 7E

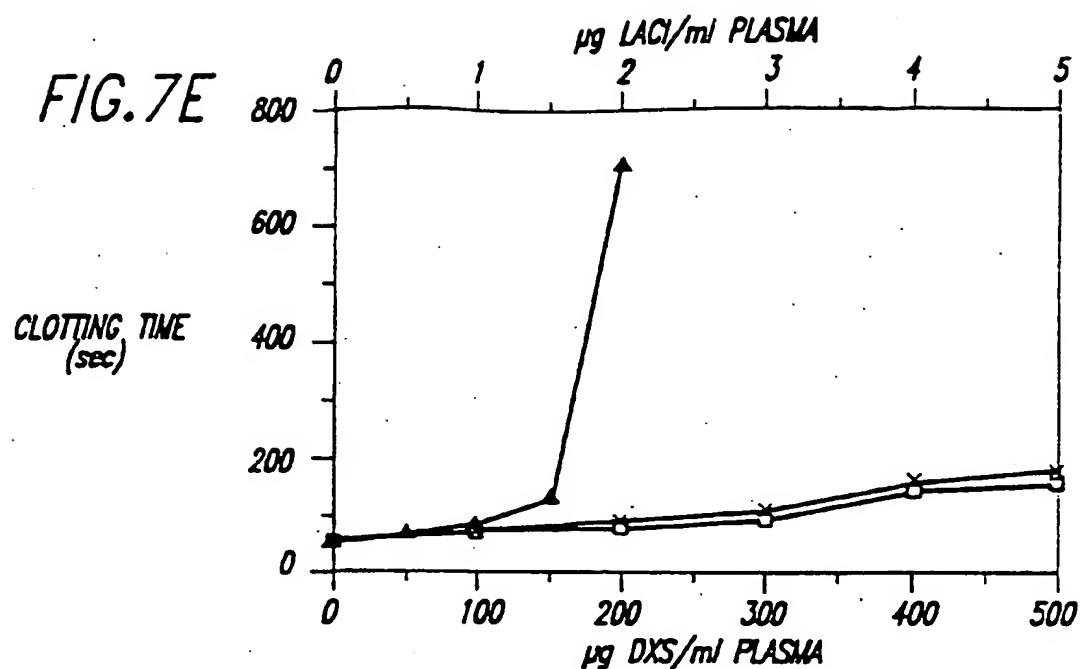
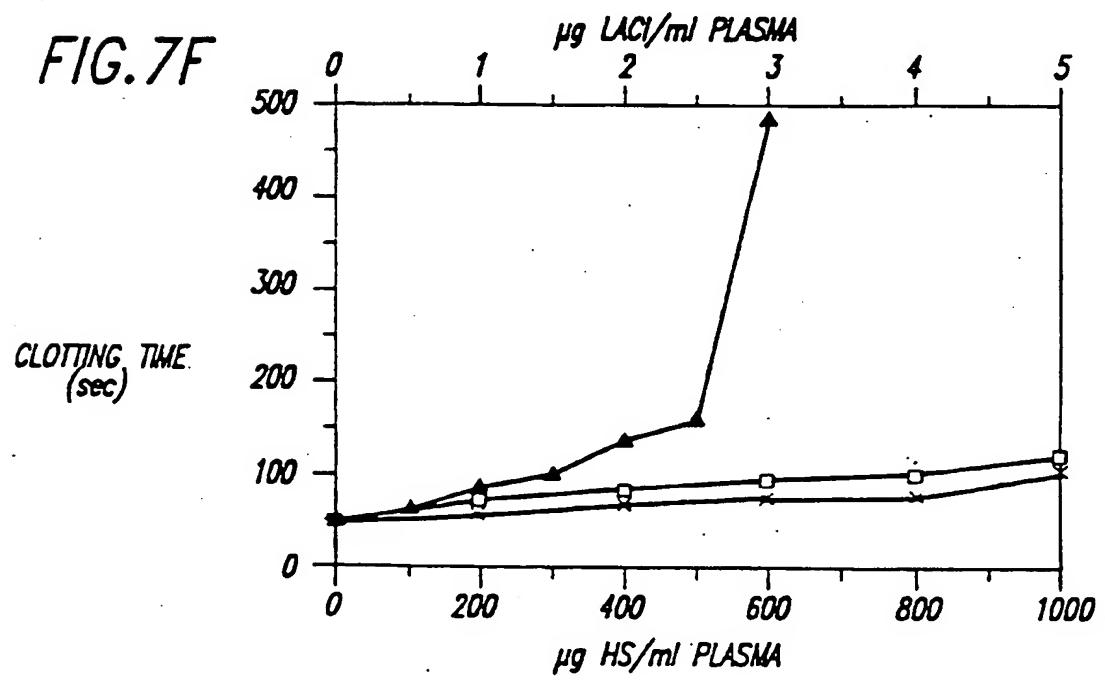


FIG. 7F





DOCUMENTS CONSIDERED TO BE RELEVANT			EP 91870132.7						
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)						
X	<p>CHEMICAL ABSTRACTS, vol. 109, no. 13, September 26, 1988, Columbus, Ohio, USA P.M. SANDSET et al. "Heparin induces release of extrinsic coagulation pathway inhibitor (EPI)." page 43, right column, abstract-no. 104 418c & Thromb. Res. 1988, 50(6), 803-13</p> <p>-----</p>	1.2	A 61 K 37/02 A 61 K 31/715						
P,A	<p><u>EP - A - 0 439 442</u> (WASHINGTON UNIVERSITY) * Claims 1,3-6 *</p> <p>-----</p>	1.7							
TECHNICAL FIELDS SEARCHED (Int. Cl.5)									
A 61 K									
<p>The present search report has been drawn up for all claims</p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 33%;">Place of search</td> <td style="width: 33%;">Date of completion of the search</td> <td style="width: 34%;">Examiner</td> </tr> <tr> <td>VIENNA</td> <td>20-11-1991</td> <td>AUGUSTIN</td> </tr> </table>				Place of search	Date of completion of the search	Examiner	VIENNA	20-11-1991	AUGUSTIN
Place of search	Date of completion of the search	Examiner							
VIENNA	20-11-1991	AUGUSTIN							
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-patent disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>& : member of the same patent family, corresponding document</p>									